The Effects of Drug and Fatty Acid Binding on Human Serum Albumin Fluorescence

An Honors Thesis (HONR 499)

by

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Abstract

In order to create a laboratory experiment focused on Fluorescence Resonance Energy Transfer for undergraduate biochemistry students at Ball State University, Human Serum Albumin, an abundant and sponge-like blood protein, was studied through the eyes of a spectrofluorometer. HSA binds very readily to many types of ligands, including drugs and fatty acids. When the lone tryptophan molecule within HSA gets excited, it fluoresces. Changing the shape of the HSA molecule by adding a fatty acid, like palmitic acid, changes the type and intensity of the fluorescence given off by the tryptophan. Adding a drug that can participate in Fluorescence Resonance Energy Transfer, like tetracycline, before adding a fatty acid allows one to quantitatively study the change in shape of the HSA molecule by calculating the exact distance between the tryptophan and the tetracycline. Overall, this research project showed promising results for this idea, but time constraints and difficulties in producing consistent data in the later stages of the project detracted from the ability to form a final unifying conclusive product.

Acknowledgements

I would like to thank Dr. Scott E. Pattison for advising me throughout this research project. From the beginning of our research days together to the very end, he continued to be supportive, open-minded, and educative. Dr. Pattison's ability to always act as a teacher and get me to answer my own questions through thought and meaningful reflection reflects the ideas of education that I can only hope to one day live up to. Thanks you, Dr. Pattison!

Introduction

Human Serum Albumin Importance:

Human Serum Albumin (HSA) accounts for over half of the protein that exists in our circulatory system. It has seven fatty acid-binding sites and two drug-binding sites, which allows it to act efficiently as a transport protein. It has been referred to as "the sponge of the circulatory system," due to its incredible ligand binding ability. It is often used in biochemical research because of those properties and how easily it is purified (2).

HSA is a globular protein with a heart-shaped tertiary structure. It consists of two domains (I and II) with largely hydrophobic resides and a third domain (III). Within a molecule of HSA lies a molecule of tryptophan. When excited by light with a wavelength of about 295nm, tryptophan fluoresces light with approximately 350nm in wavelength. This information allows us to study the effects that binding drugs or fatty acids will have on the shape of the HSA molecule. Most of the fatty acids bind to HSA in places that are far away from where a drug might bind. Even though there isn't a large amount of direct competition for binding in the same spot, there are allosteric changes caused by adding fatty acids to binding sites that are not close to the drug binding sites (2).

Inner Filter Effect Correction:

When studying fluorescence, it is important to take into consideration how much space is "wasted," so to say, in the cuvette that holds the fluorescent solution. The light shining through one side, then back out the other gets filtered or absorbed by the solution in its path. To correct for this, the raw data is put through an "inner filter effect correction" calculation. To summarize the inner filter effect, both the excitation (a) and emissions (b) lights that come in to/out of the cuvette get absorbed by the solution as they

travel through. There is also a small cubic area in the middle of the cuvette (c), in which both emission and excitation lights must be corrected for (1). Diagram A should help to visualize what this all means.

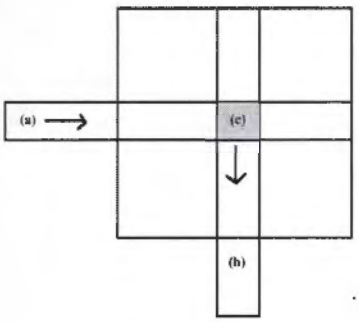


Diagram A

Visually, the inner filter effect is easy to understand. The mathematical correction for it takes a little bit more thought, however. The overall corrected fluorescence (Fl_c) can be found by multiplying the measured fluorescence (Fl_m) by both the excitation and emission filter corrections ($Filter_x$ and $Filter_m$), for a final equation that looks like this: $Fl_C = Fl_m(Filter_x * Filter_m)$ (1).

To correct for the absorbance of the excitation light, we must take into account the portion of the cuvette that sees only excitation light and the portion that sees both excitation and emission light. This can be represented as equation using the labels denoted in the diagram above: $Filter_x = Filter_{xa}Filter_{xc}$. The filter correction for a simply takes 10 the power of the length of the light beam of a (a_l) times the absorptivity value of the solution at the excitation wavelength (λ_x): $Filter_{xa} = 10^{(a_l)(\frac{absorptivity}{cm}@\lambda_x)}$. The same calculation is used for the emission's correction, except the absorptivity of the emission wavelength and the length of b would be used. The correction for the c portion of the cuvette is a little more complicated. It involves the following calculation:

$Filter_{xc} = \frac{2.3\left((c_l)(\frac{abs}{cm})_{\lambda_x}\right)}{1-10^{-c_l(\frac{abs}{cm})_{\lambda_x}}}$. The same goes for the emission filter, except again the absorptivity at the emission wavelength and the length of c would be used. The final

equation for corrected fluorescence used in this research project looks like this (1):

$$Fl_c = Fl_m 10^{(a_l)(\frac{absorptivity}{cm}@\lambda_x)} \frac{2.3\left((c_l)(\frac{abs}{cm})_{\lambda_x}\right)}{1-10^{-c_l(\frac{abs}{cm})_{\lambda_x}}} 10^{(b_l)\left(\frac{absorptivity}{cm}@\lambda_m\right)} \frac{2.3\left((c_w)(\frac{abs}{cm})_{\lambda_m}\right)}{1-10^{-c_w(\frac{abs}{cm})_{\lambda_m}}}.$$

The importance of this inner filter correction cannot be overstated. This correction allows for a more accurate look what happens to fluorescence, as the raw data gets slightly dampened by the absorption of so much light. After correcting the fluorescence measurements, a percent quench ($\%_Q$) or percent enhancement ($\%_E$) value could be more accurately calculated by finding the difference between the initial fluorescence and the final fluorescence, dividing that by the final fluorescence, and multiplying the whole thing by 100%.

Fluorescence Resonance Energy Transfer (FRET)

FRET is a phenomenon that can happen when two fluorescent molecules whose emission and excitation spectra overlap are close enough to each other that one can absorb the emitted light from the other, then emit that light out as new wavelength of light. Being able to study FRET is important for understanding the allosteric effects of binding that molecules may exhibit. In this research project, the goal was to study the FRET from the tryptophan in HSA to the newly bound tetracycline in order to see how the molecule changes as varying amounts of fatty acids (palmitic acid) were bound to the HSA. Unfortunately, the research did not get that far as time constraints and a lack of precision in the data detained the researchers from moving on (3).

Purpose

The purpose of this research was to create a new laboratory experiment for the biochemistry course (CHEM 465) at Ball State University. This laboratory experiment would have served to help students better understand FRET, through a collaborative classroom experiment in which each pair of students performs the required experiments for certain concentrations of HSA, tetracycline (TC), and palmitic acid (PA). The students would have then pooled their data together and calculated the distance between the tryptophan molecule within HSA and the bound TC molecule. If all went according to plan, the students would realize that the distance between the drug binding site and the fluorescent tryptophan changed as varying amounts of PA were bound to HSA. It is the hope that finding this information would help to give students an illustration of allosteric behavior in a relatively simple protein.

Experiments

Materials

The reagents used in these experiments were $1x10^{-5}$M HSA in a 0.1M tris buffer (with HCl) at a pH of 7.4, 0.004M PA dissolved in ethanol, and $2.7x10^{-3}$ M TC dissolved in dimethyl sulfoxide (DMSO). Solid HSA and tris were bought from Sigma-Aldrich and the TC was [borrowed from Dr. Timea Fernandez (Gerzei)'s lab] at Ball State University. All water used in these experiments was filtered with a Millipore filter. The molar absorptivity values for TC were found using a Hewlett-Packard UV-VIS spectrophotometer. All other data was recorded using Ball State's FluoroMax-3 spectrofluorometer from Jobin Yvon Horiba, Inc. The data was corrected for using Microsoft Excel and then graphed and interpreted using KaleidaGraph software.

Methods

All experiments were consistently completed using the following guidelines:

- After adding solutions to the cuvette, it was tapped against the table to remove bubbles and was wiped off using KimTech wipes to remove fingerprints.
- All solutions were equilibrated to 30°C for 30 minutes, then left in the spectrofluorometer for 10 minutes to continue equilibrating.
- Between each trial, the cuvette was rinsed thoroughly with water at least twice. Next, soap was added with a enough water to fill the cuvette about half way and it was shaken for 3 minutes. Finally, the cuvette was rinsed out at least 5 more times to ensure all soap residue was rinsed out. This washing technique was to ensure that no contamination happened between trials.

- During pipetting, the pipette tips were changed after each solution was added to the cuvette.

To determine the molar absorptivity of TC:

The HP UV-VIS machine was first blanked with nothing in the machine and set to read samples from 275-600nm. Then, 1mL of 0.1M tris buffer at a pH of 7.4 was added to the cuvette and the machine read a base-line absorbance values for the tris. Next, 20uL of $2.7x10^{-3}$ M TC in DMSO was added to the cuvette. The readings from the TC run and the tris run were subtracted and then divided by the concentration of TC in the cuvette. The absorptivity values were found for 330 and 395nm. This experiment consisted of five trials and the values were averaged in order to use them in the inner filter corrections.

To study the effects of binding TC to HSA (Experiment 1):

Each experiment used exactly 2000uL of HSA in a cuvette. For each trial, ten samples, each with a different concentration of TC, were examined. These concentrations were prepared by adding the following amounts of TC to the 2000uL of HSA: 0uL, 2uL, 4uL, 6uL, 8uL, 10uL, 15uL, 20uL, 30uL, and 40uL. Each time, the spectrofluorometer was set to excite the solution at 295nm and read from ~300- ~580nm. This gave complete emission spectra that helped to visualize the accuracy and precision the techniques used in these experiments. Furthermore, data at the 330nm and 395nm ranges were taken and analyzed to study the percent quench ($\%_Q$) that each concentration of tetracycline induced upon the HSA solution.

To study the effects of binding PA to HSA (Experiment 2):

Instead of adding TC, various amounts of 0.004M PA in ethanol were added to the solution. Since no TC was present, no inner filter corrections had to be made. The

same emission wavelengths as above were analyzed for the addition of 0uL, 10uL, 20uL, 30uL and 40uL of PA to the HSA solution. The purpose of this experiment was to study the allosteric changes involved with binding varying amounts of PA to HSA.

To study the effects of binding varying concentrations of PA in the presence of TC (Experiment 3):

The same experiment as above was run, but with varying amounts of TC present. This gave some insight on how the binding of TC affects HSA ability to change as it binds to different amounts of PA. The varying amounts of TC present were the same used as in Experiment 1, but eventually scaled back to just looking at 0uL and 40uL of TC present. The amounts of PA used in each investigation were the same as in the previous experiment.

To study the effects of binding varying concentrations of TC in the presence of PA (Experiment 4):

This experiment was to study TC's ability to quench fluorescence with varying amounts of PA present. A full TC titration (same as Experiment 1) was run under different amounts of PA present (0uL, 10uL, 15uL, 20uL, 30uL, and 40uL). The $\%_Q$ of each value was then compared to that of the other values.

To qualitatively examine the degradation of TC in the spectrofluorometer (Experiment 5):

After brainstorming reasons why the previous data wouldn't come out quantitatively consistent, an experiment to test how much light the photosensitive TC can handle while it's in the spectrofluorometer. Having taken all other precautions to keep the experiments consistent, the thought was that the TC might have been degrading as it was

being used in the experiments and that was the reason why they were not giving consistent numbers. In order to run this experiment, 2000uL of the the stock tris buffer solution and 40uL of $2.7x10^{-3}$ M TC in DMSO were added to the cuvette and a time-based acquisition experiment was run. Since light is run through a slit before it hits the cuvette, it was decided to test how much light the TC could handle as the slit width was changed. Two types of experiments were done here, both with leaving the slit open for 20 minutes and recording the intensity of the TC fluorescence at 550nm when excited at 350nm. One experiment left the slit open at 2nm, which is what the other experiments used. The other left the slit at 20nm, in order to flood the TC with light to see if it does degrade. No calculations were performed on these measurements, but the spectra were examined qualitatively.

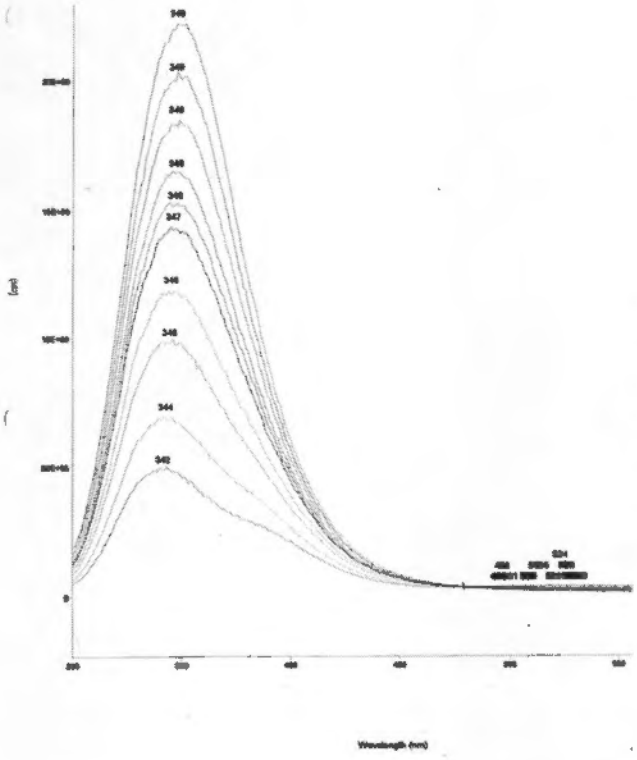
Analysis/Results

Molar Absorptivity of TC

This experiment gave consistent values for all five trials. The average value for the absorptivity of TC at 330nm was $7236 M^{-1}cm^{-1}$ and at 395nm, $4807 M^{-1}cm^{-1}$. These results helped to complete the calculation for the inner filter correction.

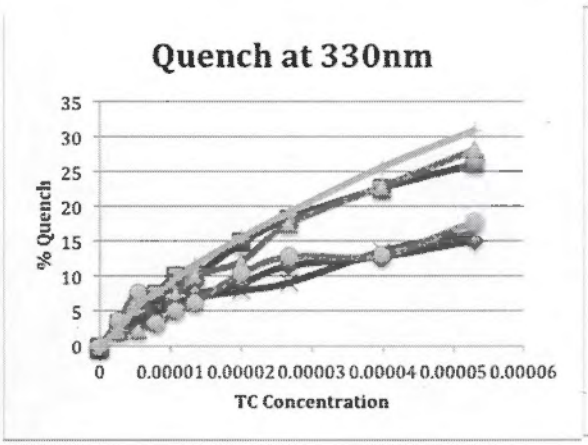
Experiment 1

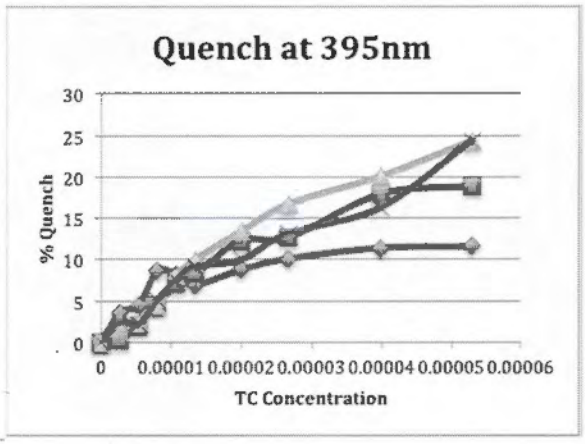
This experiment gave results that consistently showed a qualitative quench in tryptophan fluorescence. There was some inconsistency among data sets, but they fell within an acceptable range of precision for the purpose of this experiment. The graph below shows the raw data from one of these titrations graphically (increasing TC concentration from top to bottom). A clear quench can be seen as well as a blue shift in the peak wavelengths.



Over many trials, the average value for the $\%_Q$ at 330nm was found to be 22.8%. The average value for the $\%_Q$ at 395nm was found to be 18.94%. Even though TC seems to show a nearly 78% quench according to the graph above, with the inner filter correction in place, you can see that a lot of that "quench" comes from the TC absorbing the light going in to and coming out of the cuvette.

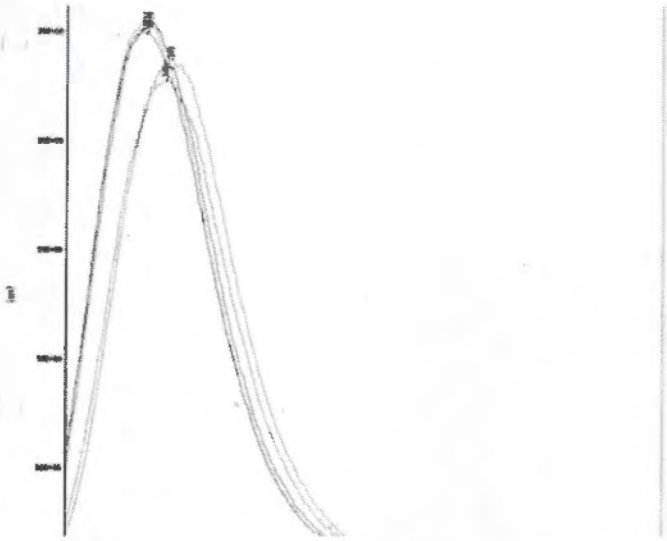
The graphs below show the $\%_Q$ for multiple data sets at 330nm and 395nm respectively.

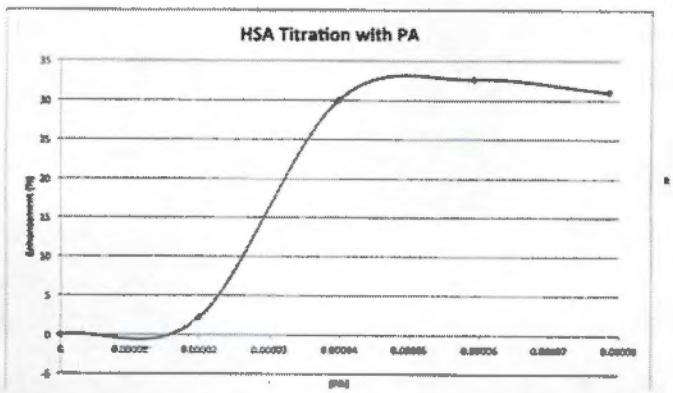




Experiment 2

As mentioned before, this experiment was to show that the addition of a fatty acid (in this case Palmitic Acid) affects the shape of the HSA molecule. As can be seen from the representative graph below (increasing PA concentration from bottom to top), HSA seemed to fluoresce in two very distinct ways. From these experiments, it was assumed that after the binding of PA in the first three to four fatty acid binding sites of HSA, there was a distinct conformation change that caused the HSA to fluoresce with more intensity and a smaller wavelength. As no TC was present, no inner filter correction was necessary, so the data in this graph is accurate.



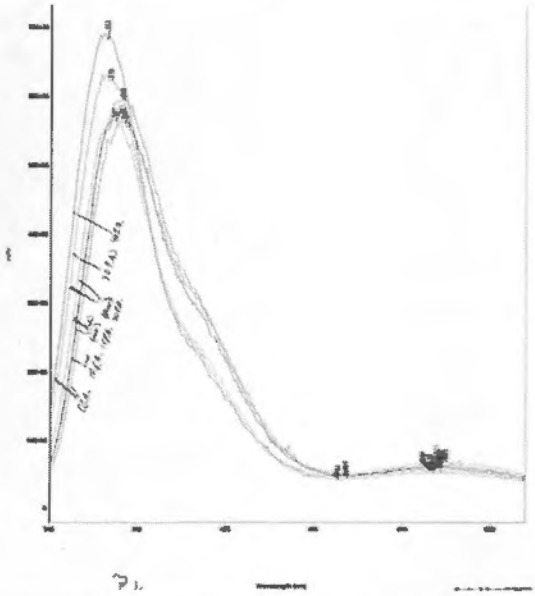


According to the data

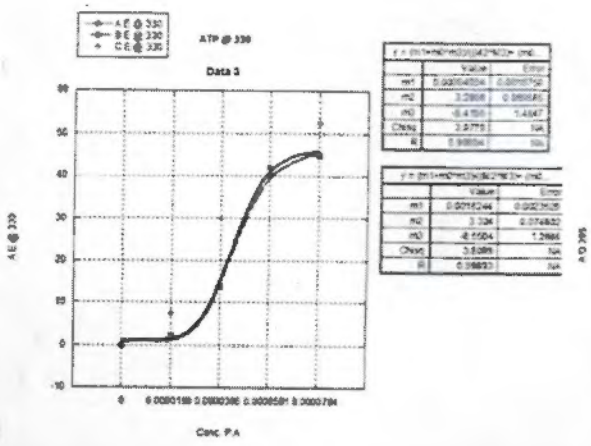
from at least four trials, the average percent enhancement ($\%_E$) for PA at 330nm was 30.8%. At 395nm, due to the blue shift, PA caused a quench in HSA fluorescence of 30.5% on average. The graph on the left represents the data at 330nm from one trial of this experiment.

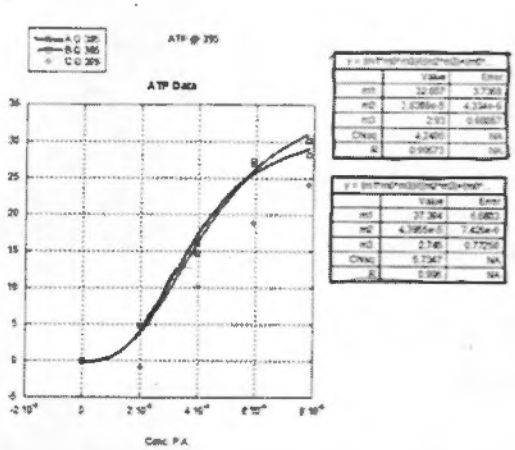
Experiment 3

The same experiment was conducted, but in the presence of TC. This was to determine how the presence of TC might affect PA's ability to change the HSA conformation. The addition of PA caused a similar conformation change, as it also involved a blue shift, as can be seen from the graph below. There was an inner filter correction associated with this data, as TC was present.



It was found that with TC present, PA enhanced fluorescence at 330nm by 44.2% on average and quenched fluorescence at 395nm by 29.1% on average. Compared to the absence of TC, this is an increase in enhancement at 330nm, and a slight decrease in quench at 395nm. The graphs below are representative of a trial done for this experiment. It can clearly be seen that there was a conformation change somewhere





between the addition of 20uL and 30uL of PA.

Experiment 4

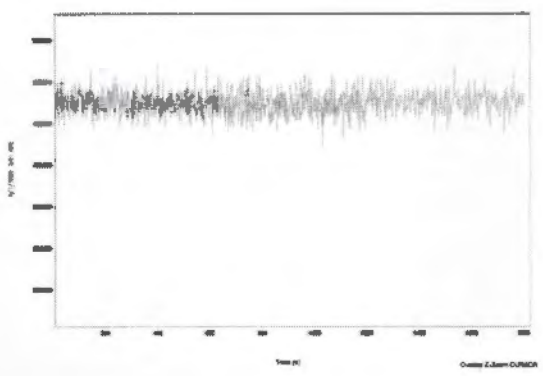
This experiment served to determine how well TC quenches fluorescence at varying concentrations of PA. This was run exactly like Experiment 1, except PA was present. With inner filter corrections, it was found that the quench at 395nm was insignificant and inconsistent, so the focus was put on the quench at 330nm. To the right is a table of the average $\%_Q$ for each amount of PA solution used in this experiment.

PA Addition	330nm
10uL	21.5%
20uL	18.65%
30uL	17.1%
40uL	20.3%

It was found that, generally, TC's ability to quench enhancement is inversely proportional to the concentration of PA. This makes sense, as we previously saw an enhancement at 330nm when PA was added with or without TC present. It is important to note, however, that not many trials of this experiment were run and there needs to be more data collected to come to a more sound conclusion here.

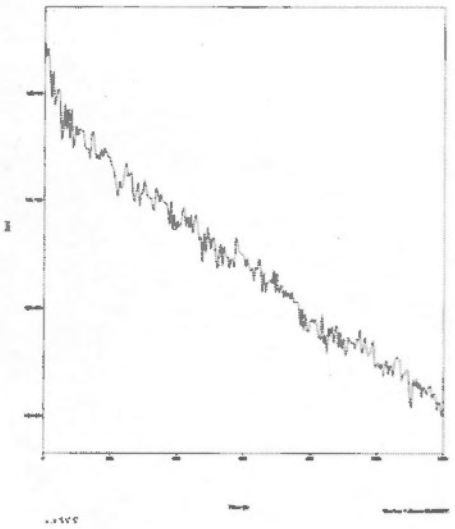
Experiment 5

The final experiment performed in this research was to determine how easily TC degrades while it's being used in the spectrofluorometer. For the experiment in which the slit was left open as wide as it was for other experiments (2nm), there did not seem to be a change in TC's fluorescence. This can be seen in the graph below.



When the slit was left open at 20nm, there was a significant change in fluorescence. This degradation can be seen in the graph below. This experiment shows that

TC is definitely photosensitive, but perhaps the amount of light it gets within normal operation of the spectrofluorometer is not enough to degrade it during experiments.



Conclusion

To conclude, this research project, while unfinished, does prove to be useful for future application. There is definite quenching when TC binds to HSA. According to the data, TC binds moderately to HSA and causes a blue shift in the fluorescence. This blue shift is indicative of a change in tryptophan's environment; specifically that it exists in a more hydrophobic environment.

No matter the environment that HSA was in, PA caused a very distinct conformation change that can be seen both qualitatively and quantitatively with the sets of data collected. This can be useful for studying allosteric changes in molecules as a result of binding and, if more work were done in this area, could be turned into a laboratory experiment.

Combined together, it can be seen that the blue shifts from TC and PA are additive. That is to say, that the blue shift when both are present is much larger than that of either one separately. It could be concluded from this that they are not the same types of blue shifts. Also, due to the only slight decline in TC quench with more PA present (likely caused by a slight decrease in TC concentration) it can be concluded that there is no competition for binding between TC and PA. If there were, there would need to be a significantly higher concentration of TC present to "cancel out" the enhancement caused by PA.

The possibility of the creation of a laboratory experiment is there, with more experimentation. From the results, it can be seen that TC is an acceptable drug to use, because it does not degrade in the spectrofluorometer during experiments.

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